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(71) Applicant *(for all designated States except US)*: CHEIL JEDANG CORPORATION [KR/KR]; 500, 5-ga, Namdaemun-ro, Chung-ku, Seoul 100-095 (KR).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: KIM, Hyun, Su [KR/KR]; Shindong-A Apt., 32-707, Gil 2-dong, Kangdong-ku, Seoul 134-766 (KR). YOO, Wang, Don [KR/KR]; Daerim Apt., 108-1001, Daebang-dong, Dongjak-ku, Seoul 156-761 (KR). NOH, Kap, Soo [KR/KR]; 242-101, Sangdo 4 dong, Dongjak-ku, Seoul 156-034 (KR). KIM, Soo, Ok [KR/KR]; Plaza Apt., 10-601, Garak-dong, Songpa-ku, Seoul 138-060 (KR). MOON, Sang, Bum [KR/KR]; Shinmiju Apt., 3-711, Kumgok-dong, Kwonsun-ku, Suwon-shi 441-460 (KR). KIM, Jong, Wan [KR/KR]; 359-75, Anam-dong, 1-ka, Sungbuk-ku, Seoul 136-071 (KR). CHUNG, Kuen, Taik [KR/KR]; Kukdong Apt., 9-302, Kwangjang-dong, Kwangjin-ku, Seoul 143-210 (KR). SHIN, Young, Chul [KR/KR]; Jukong Apt., 233-307, Banpo 2-dong, Seocho-ku, Seoul 137-042 (KR).

(74) Agents: CHOI, Hak, Hyun et al.; Peeres Building, 6th floor, 222, 3-ka, Chungjung-ro, Seodaemun-ku, Seoul 120-013 (KR).

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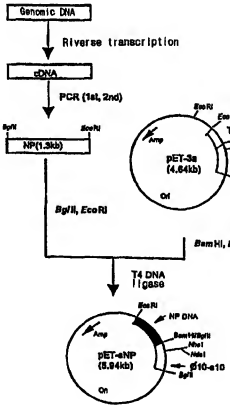
(57) Abstract

The present invention relates to a nucleocapsid gene of Seoul virus R22 strain, a recombinant expression vector for the said gene, a transformed microorganism with the said recombinant plasmid and a nucleocapsid protein expressed by the said transformant, as well as a novel pharmaceutical composition for diagnosis or prevention of haemorrhagic fever with renal syndrome caused by viruses in the *Hantavirus* genus containing the said nucleocapsid protein.



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NUCLEOCAPSID GENE OF SEOUL VIRUS R22, RECOMBINANT PLASMID, TRANSFORMED E. COLI AND DIAGNOSTIC AGENT AND VACCINE FOR HAEMORRHAGIC FEVER WITH RENAL SYNDROME

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

The present invention relates to a novel pharmaceutical composition for diagnosis or prevention of haemorrhagic fever with renal syndrome caused by viruses in the *Hantavirus* genus, as well as a nucleocapsid gene of Seoul virus R22 strain, a recombinant expression vector for the said gene, a transformed microorganism with the said recombinant plasmid and a nucleocapsid protein expressed by the said transformant.

DESCRIPTION OF THE PRIOR ART

It has been reported that there are more than ten serologically distinct hantaviruses in the *Hantavirus* genus of the family *Bunyaviridae*. Hantaan virus or Seoul virus among such hantaviruses are known as the major aetiological agents of haemorrhagic fever with renal syndrome (HFRS) in Korea. The two viruses contain a tripartite. single-stranded, negative-sense RNA species (S, M and L segments) in common. The S, M and L genome segments encode nucleocapsid protein, glycoproteins G1 and G2, and RNA polymerase, respectively. The nucleocapsid protein and the glycoproteins G1 and G2 are implicated in an immunoreaction.

To develop effective diagnostic agent and vaccines for HFRS, extensive

studies have been performed by using viruses *per se* and proteins implicated in the HFRS immunoreaction. Since the primary condition of HFRS seems to be a cold and the symptoms of HFRS are substantially similar to those suffering from the disease caused by *Leptospira* or *Rickettsia*, it is difficult to accurately diagnose the HFRS with clinical observations. The treatment of HFRS at the primary conditions would prevent such condition from turning fatal. Therefore, it is necessary to make a correct diagnosis of HFRS at the primary conditions.

The correct diagnosis of HFRS can be accomplished by using serological tests. In this assay, blood is collected from a patient twice a week and an increase of titres of anti-hantavirus antibodies is observed by indirect immunofluorescence technique or plaque reduction of neutralization antibody assay. The serological tests are accurate but require at least 7 days. Moreover, these assays are accompanied by cumbersome cell culture.

Many studies have been made to develop alternative diagnostic means of HFRS, while shortening the requirement time for diagnosis and avoiding the cumbersome process of the cell culture. Such studies are focused on the production of viral antigens which react with the anti-hantavirus antibodies in the patient blood. Nucleocapsid protein or G1 or G2 glycoproteins might be considered as the hantavirus antigen for diagnosis of HFRS. The nucleocapsid protein acts as a major antigen at the beginning of the hantavirus infection and induces a large quantity of antibodies in HFRS patients. The antibodies to the nucleocapsid protein are retained for a short duration. In this regard, the nucleocapsid protein are proper as an antigen for diagnosis of HFRS. In contrast, since the antibodies to G1 or G2 glycoproteins can be retained for many years, they are inappropriate as being indicative of the diagnosis of acute HFRS patients.

Commercially available antigens used as a diagnostic agent for HFRS are produced through cell culture or enrichment of the hantavirus on mouse brain. However, such cell culture or enrichment requires high costs and have resulted in low yields as well as high danger of viral infections among laboratory workers and animal handlers. Under the circumstances, there have been many studies to massively produce the hantavirus nucleocapsid protein through genetically recombinant technology.

Schmaljohn, etc. of *J. Gen. Virol.* (1988), **69**, 777-786 suggested expression of the nucleocapsid gene of Hantaan virus in insect cells through baculovirus expression system and potential use of the expressed nucleocapsid protein as a diagnostic antigen for HFRS. However, the expressed amount of the nucleocapsid protein is insufficient.

Wang, etc. of J. Gen. Virol. (1993), **74**, 1115-1124 and Gott, etc. of Virus Res. (1991), 19, 1-16 reported E. coli expression of the nucleocapsid gene of Hantaan virus and potential use of the expressed nucleocapsid protein for diagnosis of HFRS. However, their methods are disadvantageous because of low yield and insufficient purity. Another disadvantage is that the expressed nucleocapsid protein is generated as inclusion body.

It is commonly known that viral vaccines made from attenuated or inactivated whole virus are relatively effective. However, there are several problems in the production of vaccines using attenuated or inactivated virus. First, cultivation of the virus is usually inconvenient, accompaying cell culture. Second, it is difficult to isolate and purify the virus from cultures in high purity. Third, the possibility to obtain the intact virus is very low because the most of virus is destroyed during purification process. Fourth, the attenuation or inactivation of the purified virus for vaccine are conventionally achieved by formalin treatment or heating. This procedure might lead to denaturation of the

vaccine. Fifth, such vaccines are defective in safety which becomes the most considerable factor in production of vaccines.

A first generation vaccine for Hantaan virus has been prepared by using the isolated virus from cerebral fluid of mouse following cultivation of the virus on the mouse brain. However, it is impossible to completely remove the basic myelin protein which is known to cause certain side effect, from the cerebral fluid of mouse. Furthermore, a trace of unidentified proteins derived from mouse brain may exist in the vaccine and as a result, doubts on the safety of the vaccine can arise.

Many researchers have striven to develop cell free production system or *in vitro* expression system for preparation of the hantavirus antigenic proteins without hazardous proteins or substances. For instance, C. S. Schmaljohn, etc. elucidated nucleotide sequences and gene arrangements of M, S and L segment genomes of original Hantaan virus 76-118 and observed the preventive capability of Hamster against Hantaan virus following injection of the combined vaccinia gene with M segment encoding glycoproteins G1 and G2 into the animal body. They also reported that S segment encoding nucleocapsid protein increases the preventive capability of glycoproteins. Further, They managed to express G1, G2 and nucleocapsid protein of Hantaan virus in insect cells using baculovirus and then addressed the vaccination of animal with the crushed insect cell lysates but failed to observe the formation of neutralization antibodies derived from the nucleocapsid protein of Hantaan virus.

The nucleocapsid protein of hantavirus is a structural protein which is expressed from S segment of RNA of the virus and then exists as a complex with the RNA. Since the nucleocapsid protein constitutes most part of the viral proteins, the purification of the nucleocapsid protein from virus cultures

is convenient. However, the studies on the induction of neutralization antibodies of the hantavirus nucleocapsid protein accomplished until now are only the use of vaccinia virus as the expression vector for the nucleocapsid gene and the administration of infected cell lysates including the expressed nucleocapsid protein into animal. The purely isolated nucleocapsid protein of hantavirus has never been applied for a study on the formation of neutralization antibodies and therefore nobody has perceived the hantavirus nucleocapsid protein to be a valuable vaccine for HFRS by generating neutralization antibodies.

Meanwhile, it is very difficult to purify the glycoproteins (G1 and G2) of Hantaan virus and other viruses of the Hantavirus genus and any genetic engineering methods have not made it possible to massively produce the glyco proteins in E. coli, yeast or animal cells.

Despite that many problems may occur when vaccine is prepared from virus *per se*, all of the currently available vaccines for the prevention of HFRS have been prepared by directly culturing the virus on mouse brain or animal cell and inactivating the purified virus from the cultures with formalin.

The inventors have intensively studied to develop a method which makes it possible to massively produce the antigen for diagnosis of HFRS while ensuring the enhanced safety, diminishing costs and eliminating the tedious and troublesome process of cell culture. The inventors now attained to elucidate the full nucleotide sequence of nucleocapsid gene of Seoul virus R22 and successfully developed a novel expression system capable of massively producing the Seoul virus R22 nucleocapsid protein in a fused form of protein having extra 14 amino acids at amino terminus in E. coli and a method for purifying the nucleocapsid protein. It was now found that the purified nucleocapsid protein produced by the inventors can be used as an active

material for the desired rapid, convenient, sensitive, safe HFRS diagnostic preparation. In addition to the use of the present nucleocapsid protein for diagnosis of HFRS patients, it was also found that the present nucleocapsid protein can be also used to determine the formation of antibodies in individuals inoculated with HFRS vaccines. This finding means that the use sphere of the present nucleocapsid protein in the diagnostic preparation for HFRS is wider than that of the current available diagnostic preparations.

The inventors also found that the present nucleocapsid protein of Seoul virus is very useful in the preparation of vaccine for HFRS caused by either Seoul virus or Hantaan virus. It was reported that the vaccine prepared by inactivating Seoul virus is capable of protecting individuals from even Hantaan virus, in addition to Seoul virus (Yamanishi, et al., Vaccine (1988), 6, 278-282). This report is why the inventors selectively used the nucleocapsid protein of Seoul virus in order to prepare vaccine for prevention of HFRS caused by both Seoul virus and Hantaan virus.

In addition, the inventors found that the vaccine prepared by using the present nucleocapsid protein is considerably superior over the current available vaccines in terms of its efficacy and safety.

SUMMARY OF THE INVENTION

The present invention provides a nucleocapsid gene of Seoul virus R22 having the following nucleotide sequence:

```
          10         20         30         40         50
          |          |          |          |          |
ATGGCAACTATGGAGGAAATCCAGAGAGAAATCAGTGCTCACGAGGGGCA

          60         70         80         90         100
          |          |          |          |          |
GCTTGTGATAGCACGCCAGAAAGTCAAGGATGCAGAAAAGCAGTATGAAA

          110        120        130        140        150
          |          |          |          |          |
AGGATCCTGATGACTTCAACAAGAGGGCACTGCATGATCGGGAGAGTGTC

          160        170        180        190        200
          |          |          |          |          |
GCAGCTTCAATACAATCAAAAATTGATGAATTGAAGCGCCAACTTGCCGA

          210        220        230        240        250
          |          |          |          |          |
CAGGATTGCAGCAGGGAAGAATATTGGGCAAGACCGGGATCCTACAGGGG

          260        270        280        290        300
          |          |          |          |          |
TAGAGCCGGGTGATCATCTCAAAGAGAGATCAGCACTAAGCTATGGGAAT

          310        320        330        340        350
          |          |          |          |          |
ACACTGGACCTGAATAGTCTTGACATTGATGAACCTACAGGACAGACAGC

          360        370        380        390        400
          |          |          |          |          |
TGATTGGTTGACCATAATTGTCTATCTGACTTCATTTGTGGTCCTGATCA

          410        420        430        440        450
          |          |          |          |          |
TCTTAAAGGCACTGTACATGTTAACAACAAGAGGCAGGCAGACTTCAAAG

          460        470        480        490        500
          |          |          |          |          |
GACAACAAAGGGATGAGGATCAGATTCAAGGATGACAGCTCATATGAGGA
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```
          510         520         530         540         550
           |           |           |           |           |
TGTCAATGGAATCAGAAAACCCAAACATCTGTATGTGTCAATGCCAAACG

          560         570         580         590         600
           |           |           |           |           |
CCCAATCCAGCATGAAGGCTGAAGAGATAACACCTGGAAGATTCCGCACT

          610         620         630         640         650
           |           |           |           |           |
GCAGTATGTGGGCTATATCCTGCACAGATAAAGGCAAGGAACATGGTGAG

          660         670         680         690         700
           |           |           |           |           |
CCCTGTCATGAGTGTAGTTGGGTTTTTGGCACTGGCAAAAGATTGGACAT

          710         720         730         740         750
           |           |           |           |           |
CTAGAATTGAAGAATGGCTTGGTGCACCCTGCAAGTTTATGGCGGAATCT

          760         770         780         790         800
           |           |           |           |           |
CCAATTGCTGGGAGTTTATCTGGGAATCCCGTGAATCGTAACTATATCAG

          810         820         830         840         850
           |           |           |           |           |
ACAGAGACAAGGTGCACTTGCAGGGATGGAGCCAAAGGAATTTCAAGCCC

          860         870         880         890         900
           |           |           |           |           |
TCAGGCAACATGCAAAGGATGCTGGGTGTACACTGGTTGAGCATATTGAG

          910         920         930         940         950
           |           |           |           |           |
TCACCATCATCAATATGGGTGTTTGCTGGGGCCCCTGATAGGTGTCCACC

          960         970         980         990        1000
           |           |           |           |           |
AACATGTTTGTTTGTCGGAGGGATGGCTGAGTTAGGTGCCTTCTTTTCTA
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```
          1010        1020        1030        1040        1050
           |           |           |           |           |
TACTTCAGGATATGAGGAACACAATCATGGCTTCAAAAACTGTGGGCACA

          1060        1070        1080        1090        1100
           |           |           |           |           |
GCTGATGAAAAGCTTCGAAAGAAATCATCATTCTATCAATCATACCTCAG

          1110        1120        1130        1140        1150
           |           |           |           |           |
ACGCACACAATCAATGGGAATACAACTGGACCAGAGGATAATTGTTATGT

          1160        1170        1180        1190        1200
           |           |           |           |           |
TTATGGTTGCCTGGGGAAAGGAGGCAGTGGACAACTTTCATCTCGGTGAT

          1210        1220        1230        1240        1250
           |           |           |           |           |
GACATGGACCCAGAGCTTCGTAGCCTGGCTCAGATCCTGATTGACCAGAA

          1260        1270        1280        1290
           |           |           |           |
AGTAAAAGAAATCTCGAACCAGGAACCTTTGAAACTCTAA
```

Further, the present invention provides a recombinant expression plasmid including the nucleocapsid gene of Seoul virus R22 of the present invention.

Furthermore, the present invention provides a transformed *Escherichia coli* with the recombinant expression plasmid of the present invention.

Furthermore, the present invention provides a method for producing a fused form of nucleocapsid protein of Seoul virus R22 in high yield which comprises culturing the transformed *E. coli* of the present invention on an appropriate medium to express the said nucleocapsid protein, and isolating and purifying the expressed nucleocapsid protein from the cultures.

Furthermore, the present invention provides a highly purified fused form of nucleocapsid protein of Seoul virus R22 having the following amino acid sequence:

```
          10        20        30        40        50
          |         |         |         |         |
MASMTGGQQMGRGSMATMEEIQREISAHEGQLVIARQKVKDAEKQYEKDP

          60        70        80        90        100
          |         |         |         |         |
DDFNKRALHDRESVAASIQSKIDELKRQLADRIAAGKNIGQDRDPTGVEP

          110       120       130       140       150
          |         |         |         |         |
GDHLKERSALSYGNTLDLNSLDIDEPTGQTADWLTIIVYLTSFVVLIILK

          160       170       180       190       200
          |         |         |         |         |
ALYMLTTRGRQTSKDNKGMRIRFKDDSSYEDVNGIRKPKHLYVSMPNAQS

          210       220       230       240       250
          |         |         |         |         |
SMKAEEITPGRFRTAVCGLYPAQIKARNMVSPVMSVVGFLALAKDWTSRI

          260       270       280       290       300
          |         |         |         |         |
EEWLGAPCKFMAESPIAGSLSGNPVNRNYIRQRQGALAGMEPKEFQALRQ

          310       320       330       340       350
          |         |         |         |         |
HAKDAGCTLVEHIESPSSIWVFAGAPDRCPPTCLFVGGMAELGAFFSILQ

          360       370       380       390       400
          |         |         |         |         |
DMRNTIMASKTVGTADEKLRKKSSFYQSYLRRTQSMGIQLDQRIIVMFMV

          410       420       430       440
          |         |         |         |
AWGKEAVDNFHLGDDMDPELRSLAQILIDQKVKEISNQEPLKL
```

Furthermore, the present invention provides a pharmaceutical composition for diagnosis of HFRS comprising the nucleocapsid protein of the present invention in combination with a pharmaceutically acceptable carrier or excipient.

Furthermore, the present invention provides a use of the pharmaceutical composition comprising the nucleocapsid protein of the present invention in determination on the formation of the antibodies to hantaviruses in a subject administered with a vaccine for HFRS.

Furthermore, the present invention provides a diagnostic formulation for HFRS comprising the nucleocapsid protein of the present invention coated on a 96-well plate.

Furthermore, the present invention provides a diagnostic formulation for HFRS comprising the nucleocapsid protein of the present invention adsorbed on a nitrocellulose membrane.

Furthermore, the present invention provides a vaccine for prevention of HFRS comprising the nucleocapsid protein of the present invention as an active ingredient in combination with a pharmaceutically acceptable adjuvant. The adjuvant may be selected from a group consisting of aluminium hydroxide gel, chimerosal and tablet gelatin, and a mixture thereof.

BRIEF DESCRIPTION OF THE DRAWING

Fig. 1 shows the sites of the chemically snythesized on the nucleocapsid gene of Seoul virus strain R22 nucleocapsid gene.

Fig. 2 is a schematic construction diagram showing a recombinant

expression plasmid pET-sNP containing Seoul virus R22 nucleocapsid gene.

Fig. 3 is a flow diagram showing a purification process for Seoul virus R22 nucleocapsid protein expressed by transformant *E. coli* BL containing plasmid pET-sNP.

Fig. 4 is (a) polyacrylamide gel electrophoresis and (b) western blots of Seoul virus R22 nucleocapsid protein expressed by transformant *E. coli* BL containing plasmid pET-sNP.

Fig. 5 is western blots of Seoul virus R22 nucleocapsid protein expressed by transformant *E. coli* BL containing plasmid pET-sNP using (a) human patient blood and (b) anti-nucleocapsid protein monoclonal antibodies.

Fig. 6 is a photograph showing the reaction of patient serum (P) or healthy subject serum (N) on nitrocellulose membrane adhered by various concentrations of Seoul virus R22 nucleocapsid protein.

Fig. 7 is western blots of nucleocapsid proteins expressed by several transformant *E. coli* strains containing different vectors.

DETAILED DESCRIPTION OF THE INVENTION

The present invention was accomplished through the amplification of the fully elucidated nucleotide sequence of a nucleocapsid gene of Seoul virus R22, the preparation of a recombinant expression plasmid, the production of a transformed *E. coli*, the cultivation of the transformant, the isolation and purification of the expressed nucleocapsid protein, and the evaluation of the diagnostic agent and vaccine comprising the nucleocapsid protein on efficacy and safety.

A large amount of the nucleocapsid gene of Seoul virus R22 to be cloned into an expression vector was prepared through twice polymerase chain reactions using three primers having the following nucleotide sequences, respectively:

NP 1 (17MER): 5'-TAGTAGTAGACTCCCTA-3'

NP 2 (23MER): 5'-CCAGATCTATGGCAACTATGGAG-3'
***Bgl* II**

NP 3 (22MER): 5'-GGAATTCTTAGAGTTTCAAAGG-3'
***EcoR* I**

The first polymerase chain reaction used the designated NP1 and NP3 primers to amplify the nucleocapsid gene of Seoul virus R22. The second polymerase chain reaction used the designated NP2 and NP3 primers to introduce recognization sites for *Bgl*II and 20 *Eco*RI into the nucleocapsid gene, which will be thereby cloned into a commercially available expression vector pET-3a (Stratagene, cat#211621).

Since the vector pET-3a used for the cloning of the nucleocapsid gene of the present invention includes s10 sequence, the nucleocapsid protein is expressed as a form of a fused protein having additional 14 amino acids at the amino terminus.

Fig 2. illustrates a whole process for constructing a recombinant plasmid for expression of the nucleocapsid gene of the present invention by cloning the said gene into the vector pET-3a. The resulting recombinant

plasmid was designated as pET-sNP.

A novel transformed microorganism was produced by introducing the plasmid pET-sNP of the present invention into a host cell, *E. coli* BL21(DE3) (Stratagene, cat#211621) through electric shock and was proved as being capable of massively producing the desired nucleocapsid protein. This microorganism, referred to as E. coli BL(pET-sNP), was deposited with the Korean Culture Center of Microorganisms under the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure on September 10, 1997 and the accession number was designated as KFCC-10111.

The nucleotide sequencing of the nucleocapsid gene of Seoul virus R22 was carried out by using Auto Reader Sequencing Kit (Pharmacia Co., cat#27-2690-20, cat#18-1033-13) in accordance with a known dideoxy method (PNAS 74, p5463, 1977). Then, the recombinant expression plasmid pET-sNP was used as a template and the synthetic fragments having the following nucleotide sequences were used as a primer:

P1: 5' GGCTAGCATGACTGGTGG-3'

P2: CTATCTGACTTCATTTGTGG

P3: ACCCAAACATCTGTATGTG

P4: GTITATCTGGGAATCC

P5: GAGGAACACAATCATGG

P6: CCATTATTATCATAGC

P7: TGATTGTGTGCGTCTGAGGT

P8: ATTCTTCAATTCTAGATGTC

P9: CAGTGCCTTTAAGATGAT

In order to ensure the accuracy of the nucleotide sequencing, the nucleocapsid gene was twice analysed from 5'-end to 3'-end and, reversely, from 3'-end to 5'-end. The elucidated nucleotide sequence of the nucleocapsid gene of Seoul virus R22 in comparison with the known nucleotide sequence of the nucleocapsid gene of Seoul virus SR11(Virology 176, pp114-125, 1990) is as follows:

```
          10        20        30        40        50
ATGGCAACTATGGAGGAAATCCAGAGAGAAATCAGTGCTCACGAGGGGCA   R-22.SEQ
ATGGCAACTATGGAAGAAATCCAGAGAGAAATCAGTGCTCACGAGGGGCA   SR-11.SEQ

          60        70        80        90       100
GCTTGTGATAGCACGCCAGAAAGTCAAGGATGCAGAAAAGCAGTATGAAA   R-22.SEQ
GCTTGTGATAGCACGCCAGAAGGTCAAGGATGCAGAAAAGCAGTATGAGA   SR-11.SEQ

         110       120       130       140       150
AGGATCCTGATGACTTCAACAAGAGGGCACTGCATGATCGGGAGAGTGTC   R-22.SEQ
AGGATCCTGATGACTTAAACAAGAGGGCACTGCATGATCGGGAGAGTGTC   SR-11.SEQ

         160       170       180       190       200
GCAGCTTCAATACAATCAAAAATTGATGAATTGAAGCGCCAACTTGCCGA   R-22.5SEQ
GCAGCTTCAATACAATCAAAAATTGATGAATTGAAGCGCCAACTTGCCGA   SR-11.SEQ

         210       220       230       240       250
CAGGATTGCAGCAGGGAAGAATATGGGGCAAGACCGGGATCCTACAGGGG   R-22.SEQ
CAGGATTGCAGCAGGGAAGAAGATGGGGCAGGACCGGGATCCTACAGGGG   SR-11.SEQ

         260       270       280       290       300
TAGAGCCGGGTGATCATCTCAAAGAGAGATCAGCACTAAGCTATGGGAAT   R-22.SEQ
TAGAGCCAGGTGATCATCTTAAAGGAGAGATCAGCACTAAGCTAGGGAAT   SR-11.SEQ

         310       320       330       340       350
ACACTGGACCTGAATAGTCTTGACATTGATGAACCTACAGGACAGACAGC   R-22.SEQ
ACACTGGACCTGAATAGTCTTGACATTGATGAACCTACAGGACAGACAGC   SR-11.SEQ

         360       370       380       390       400
TGATTGGTTGACCATAATTGTCTATCTGACTTCATTTGTGGTCCTGATCA   R-22.SEQ
TGATTGGGTGACCATAATTGTCTATCTGACATTCATTGGTGGTCCGGATCA   SR-11.SEQ
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          410        420        430        440        450
TCTTMAAGGCACTGTACATGTTAACAACAMGAGGCAGGCAGACTTCAAAG   R-22.SEQ
TCTTCAAGGCACTGTACATGTTAACAACACGAGGCAGGCAGACTTCAAAG   SR-11.SEQ

          460        470        480        490        500
GACAACAAMGGGATGAGGATCAGATTCAAGGATGACAGCTCATATGAGGA   R-22.SEQ
GACAACAACGGGATGAGGATCAGATTCAAGGATGACAGCTCATATGAGGA   SR-11.SEQ

          510        520        530        540        550
TGTCAATGGAATCAGAAAMCCCAAACATCTGTATGTGTCAATGCCAAACG   R-22.SEQ
TGTCAATGGAATCAGAAACCCCAAACATCTGTATGTGTCAATGCCAAACG   SR-11.SEQ

          560        570        580        590        600
CCCAATCCAGCATGAAGGCTGAAGAGATAACACCCTGGAAGATTCCGCACT  R-22.SEQ
CCCAATCCAGCATGAAGGCTGAAGAGATAACACCAGGAAGATTCCGCACT   SR-11.SEQ

          610        620        630        640        650
GCAGTATGTGGCCTATATCCTGCACAGATAAAGGCAAGGAAGATGGTGAG   R-22.SEQ
GCAGTATGTGGMCTATATCCTGCACAGATAAAGGCAAGGAAGATGGTMAG   SR-11.SEQ

          660        670        680        690        700
CCCTGTCATGAGTGTAGTTGGGTTTTTGGCACTGGCAAAAGATTGGACAT   R-22.SEQ
CCCTGTCATGAGTGTAGTTGGGTTGTTGGCACTGGCAAAAGAGTGGACAT   SR-11.SEQ

          710        720        730        740        750
CTAGAATTGAAGAATGGCTTGGTGCACCCTGCAACTTTATGGCGGAATCT   R-22.SEQ
CAAGAATTGAAGAATGGCTGGGTGCACCCTGCAACTTCATGGCGGAGTCT   SR-11.SEQ

          760        770        780        790        800
CCAATTGCTGGGAGTTTATCTGGGAATCCGGTGAATCGTAACTATATCAG   R-22.SEQ
CTTATTGCCGGGAGTTTATCTGGGAATCCATGTGAATCGTCACTATATCAG  SR-11.SEQ
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          810        820        830        840        850
ACAGAGACAAGGTGCACTTGCAGGGATGGAGCCAAAGGAATTTCAAGCCC   R-22.SEQ
ACAGAGACAAGGTGCACTTGCAGGGATGGAGCCAAAGGAATTTCAAGCCC   SR-11.SEQ

          860        870        880        890        900
TCAGGCAACATTCCAAAGGATGCTGGTTGTACACTTGTTGAATCATATTGAG   R-22.SEQ
TCAGGCAACATTTCAAAGGATGCTGGATGTACACTAGTTGAATCATATTGAG   SR-11.SEQ

          910        920        930        940        950
TCACCATCATCAATATGGGTGTTTGCTGGGGCCCCTGATAGGTGTCCACC   R-22.SEQ
TCACCATCATCAATATGGGTGTTTGCTGGGGCCCCTGATAGGTGTCCACC   SR-11.SEQ

          960        970        980        99        1000
AACATGTTTGTTTGTCGGAGGGATGGCTGATTTAGGTGCCTTCTTTTCTA   R-22.SEQ
AACATGTTTGTTTGTCGGAGGGATGGCTGAATTAGGTGCCTTCTTTTCTA   SR-11.SEQ

          1010       1020       1030       1040       1050
TACTTCAGGATATGAGGAACACAATCATGGCTTCAAAAACTGTGGGCACA   R-22.SEQ
TACTTCAGGATATGAGGAACACAATCATGGCTTCAAAAACTGTGGGCACA   SR-11.SEQ

          1060       1070       1080       1090       1100
GCTGATGAAAAGCTTCGAAAGAAATCATCATTCTATCAATCATACCTCAG   R-22.SEQ
GCTGATGAAAAGCTTCGAAAGAAATCATCATTCTATCAATCATACCTCAG   SR-11.SEQ

          1110       1120       1130       1140       1150
ACGCACACAATCAATGGGAATACAACTGGACCAGAGGATAATTGTTATGT   R-22.SEQ
ACGCACACAATCAATGGGAATACAACTGGACCAGAGGATAATTGTTATGT   SR-11.SEQ

          1160       1170       1180       1190       1200
TTATGGTTGCCTGGGGAAAGGAGGCAGTGGACAACTTTCATCTCGGTGAT   R-22.SEQ
TTATGGTTGCCTGGGGAAAGGAGGCAGTGGACAACTTTCATCTCGGTGAT   SR-11.SEQ
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          1210       1220       1230       1240       1250
GACATGGATCCAGAGCTTCGTAGCCTGGCTCAGATCTTGATTGACCAGAA    R-22.SEQ
GACATGGATCCAGAGCTTCGTAGCCTGGCTCAGATCTTGATTGACCAGAA    SR-11.SEQ

          1260       1270       1280       1290
AGTTAAAGAAATCTCTCAACCAGGAACCTTTTGAAATTTTAA            R-22.SEQ
AGTTGAAGGAAATCTCTCAACCAGGAACCTTATGAAATTTTAA            SR-11.SEQ
```

in which the reversed A, T, G, and C indicate the different nucleotides between nucleotide sequences of nucleocapsid genes of Seoul virus R22 and Seoul virus SR11. A comparison of the two nucleotide sequences reveals that 1241 nucleotides are same and thus the identity is calculated as 96.2%. In addition, upon comparison of the two presumed amino acid sequences from the Seoul virus R22 and R11 nucleotide sequences as illustrated below, it reveals that 423 amino acids are same and thus the identity is calculated as 98.6%:

```
          10         20         30         40         50
MATMEEIQREISAHEGQLVIARQKVKDAEKQYEKDPDDTNKRALHDRESV    R-22.PRO
MATMEEIQREISAHEGQLVIARQKVKDAEKQYEKDPDDINKRALHDRESV    SR-11.PRO

          60         70         80         90         100
AASIQSKIDELKRQLADRIAAGKNIGQDRDPTGVEPGDIHLKERSALSYGN    R-22.PRO
AASIQSKIDELKRQLADRIAAGKNIGQDRDPTGVEPGDIHLKERSALSYGN    SR-11.PRO

          110        120        130        140        150
TLDLNSLDIDEPTGQTADWLTIIVYLTSFVVEIILKALYMLTTRGRQTSK    R-22.PRO
TLDLNSLDIDEPTGQTADWLTIIVYLTSFVVEIILKALYMLTTRGRQTSK    SR-11.PRO
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          160        170        180        190        200
DNKGMRIRFKDDSSYEDVNGIRKPKHLYVSMPNAQSSMKAEEITPGRFRT   R-22.PRO
DNKGMRIRFKDDSSYEDVNGIRKPKHLYVSMPNAQSSMKAEEITPGRFRT   SR-11.PRO

          210        220        230        240        250
AVCGLYPAQIKARNMVSPVMSVVGFLALAKDWTSRIEEWLGAPCKFMAES   R-22.PRO
AVCGLYPAQIKARNMVSPVMSVVGFLALAKDWTSRIEEWLGAPCKFMAES   SR-11.PRO

          260        270        280        290        300
EIAGSLSGNPVNRNYIRQRQGALAGMEPKEFQALRQHAKDAGCTLVEHIE   R-22.PRO
TIAGSLSGNPVNRDYIRQRQGALAGMEPKEFQALRQHSKDAGCTLVEHIE   SR-11.PRO

          310        320        330        340        350
SPSSIWVFAGAPDRCPPTCLFVGGMAELGAFFSILQDMRNTIMASKTVGT   R-22.PRO
SPSSIWVFAGAPDRCPPTCLFVGGMAELGAFFSILQDMRNTIMASKTVGT   SR-11.PRO

          360        370        380        390        400
ADEKLRKKSSFYQSYLRRTQSMGIQLDQRIIVMFMVAWGKEAVDNFHLGD   R-22.PRO
ADEKLRKKSSFYQSYLRRTQSMGIQLDQRIIVMFMVAWGKEAVDNFHLGD   SR-11.PRO

          410        420        430
DMDPELRSLAQILIDQKVKEISNQEPTMKL                        R-22.PRO
DMDPELRSLAQILIDQKVKELSNQEPAMKL                        SR-11.PRO
```

in which the reversed characters indicate the different amino acids between amino acid sequences of nucleocapsid proteins from Seoul virus R22 and SR11.

The fragments which serve as a primer for sequencing the nucleocapsid gene of Seoul virus R22 were chemically synthesized on the basis of the known nucleotide sequence of the nucleocapsid gene of Seoul virus SR-11. Fig. 1 shows the location of the chemically synthesized primers on the nucleocapsid gene of Seoul virus R22.

The culture of the transformed E. coli of the present invention can be performed according to methods conventionally known in the art of a genetic engineering technology. The high purification of the expressed nucleocapsid protein was accomplished through ammonium sulfate precipitation, gel filtration and phenyl sepharose column chromatography. As illustrated in Fig. 3, subsequent polyacrylamide gel electrophoresis and western blotting were carried out to affirm the purified nucleocapsid protein. The results of polyacrylamide gel electrophoresis and western blotting shown in Figs. 4 and 5, respectively, prove that the purified substance corresponds to the nucleocapsid protein. In addition, the purity of the nucleocapsid protein was evaluated as at least 90% from the result of polyacrylamide gel electrophoresis.

The purified nucleocapsid protein of the present invention includes additional 14 amino acids(Met-Ala-Ser-Met-Thr-Gly-Gly-Gln-Gln-Met-Gly-Arg-Gly-Ser) at the N-terminus as compared to the corresponding naturally occurred nucleocapsid protein. The additional amino acid sequence is derived from s10 leader sequence located at the front of *Bam*HI site, cloning site of plasmid pET-3a. The molecular weight of the present nucleocapsid protein was measured as approximately 50 kDa.

An alternative recombinant plasmid, designated as pKK-sNP, for expression of the nucleocapsid protein gene of Seoul virus R22 was constructed by the inventors. The construction of the plasmid pKK-sNP is for the purpose of inspecting the influence of a cloning vector on the expression

of the nucleocapsid protein gene of Seoul virus R22. The procedure for constructing the plasmid pKK-sNP was similar to that of the plasmid pET-sNP except that pKK223-3 having the strong *tac* promoter (Pharmacia Biotech, cat#27-4935-01) was used as a cloning vector instead of pET-3a. It was found that the pET-3a express the nucleocapsid protein of Seoul virus R22 much higher than the pKK223-3. It is comprehended that such result is because the fused form of the nucleocapsid protein with additional 14 amino acids at the N-terminus allows the expressed nucleocapsid protein to be more stable in E. coli or because s10 leader sequence existing in pET-3 plays a role in increasing either the transcription efficiency of the cloned nucleocapsid protein gene or the translation efficiency in the protein synthesis from the transcript mRNA.

For the evaluation of the nucleocapsid protein expression capability of the plasmid pET-sNP of the present invention, another plasmid, designated as pET-NP was constructed by cloning the nucleocapsid gene of prototype Hantaan virus 76-118 (Virology 155, pp633-643, 1986) into pET-3a in accordance with the same procedure as used in the construction of pET-sNP of the present invention. The two expressed nucleocapsid proteins of Seoul virus R22 and Hantaan virus 76-118 were separately detected by western blotting using monoclonal antibodies to the Hantaan virus nucleocapsid protein. The results are shown in Fig. 7. It can be seen that the expressed Seoul virus R22 nucleocapsid protein is twice as high as the expressed Hantaan virus 76-118 nucleocapsid protein. The results are likely attributed to the higher stability of the Seoul virus R22 nucleocapsid protein than that of the Hantaan virus 76-118 nucleocapsid protein.

An assay on a diagnostic efficacy of the Seoul virus R22 nucleocapsid protein of the present invention was performed according to conventional methods. The nucleocapsid protein coated on 96-well plate or nitrocellulose

membrane was reacted with patient serum or vaccinee serum. In order to ensure the accuracy of the assay, the blood collected from the patient was re-assayed by an indirect immunofluorescence antibody assay. The results are shown in Fig. 6. It can be seen that the patient serum was accurately detected by both of the Seoul virus R22 nucleocapsid proteins coated on 96-well plate or nitrocellulose membrane. Also it was appreciated that the Seoul virus R22 nucleocapsid protein can be used to examine the formation of the antibodies in vaccinated subjects with HFRS vaccines.

The purified nucleocapsid protein of Seoul virus R22 of the present invention is mixed with conventional adjuvants, especially aluminium hydroxide gel, prior to its use as a formulated vaccine for HFRS. The preferred amount of the aluminium hydroxide gel used is less than 0.625 mg/ 0.5 ml of vaccine solution. In addition to the adjuvant, chimerosal and purified gelatin may be used as a preservative in amount of 0.01% (w/v) and a stabilizing agent in amount of 0.02% (w/v), respectively. Also, 0.01% (w/v) of Tween 80 can be added.

The invention will now be explained in details with reference to the following illustrative Examples.

Example 1

Isolation of genomic RNA from Seoul virus R22 and synthesis of cDNA

100 μl of culture solution of Seoul R22 virus was mixed with 400 μl of Solution A (4.2 M guanidine isothiocyanate, 25 ml Tris-HCl, pH 8.0, 0.5% Sarkosyl, 0.7% β-mercapto ethanol) and then with 50 μl of Solution B (1 M Tris-HCl, pH 8.0, 0.1 M EDTA, 10% SDS). After incubation of the resuting solution at 65℃ for 5 minutes, it was mixed with an equal volume of a mixed solution of phenol and chloroform (1:1). After another incubtion at 6

5℃ for further 30 minutes, the mixture was centrifuged. The supernatant was taken, and the remaining solution was mixed with further 300 μl of Solution A, then incubated at 65℃ for 5 minutes. Following centrifugation of the solution, the supernatant was taken and combined with the first supernatant. The total supernatant was extracted with a mixed solution of phenol and chloroform (1:1) and then with chloroform. The extracted solution was mixed with 1/10 volume of 3 M sodium acetate and two volumes of isopropyl alcohol and the resulting mixture was kept at -20℃ for 16 hours. The solution was then centrifuged at 12,000 rpm for 15 minutes, and the precipitate was washed with 70% ethyl alcohol twice and dried. The dried precipitate was dissolved in 10 μl of sterilized distilled deionized water which does not contain ribonuclease at all and the resulting solution was kept at 4℃. 5 μl of the purified genomic RNA was used to synthesize cDNA. The synthetic reaction was carried out at 37℃ for 1 hour under the conditions indicated in Table 1 below.

Table 1

Tris-HCl, pH 8.4	10 mM
KCl	50 mM
$MgCl_2$	7 mM
4 dNTPs	1 mM
RNase inhibitor	1 U/μl
Random primer	50 pmol
AMV reverse transcriptase	12 Units

Example 2

Amplification of nucleocapsid gene by the polymerase chain reaction

The nucleocapsid gene to be cloned into a known plasmid pET-3a was amplified by the polymerase chain reaction (PCR) with the primers illustrated above. The DNA Thermal Cycler 480 (Perkin Elmer) was used for the

PCR. The reaction conditions and procedures are described in Table 2 below.

Table 2

Composition for PCR	Tris-HCl, pH 8.4	10 mM
	KCl	50 mM
	$MgCl_2$	2.5 mM
	4 dNTPs	0.2 mM
	Primers	50 pmol
	Taq polymerase	2.5 Units
Procedures for PCR	92℃(15 sec)→46℃(2 min)→72℃(3 min)	20 cycles
	92℃(15 sec)→46℃(2 min)→72℃(3.5 min)	15 cycles
	92℃(15 sec)→46℃(2 min)→72℃(10 min)	1 cycle

5 μl of cDNA solution prepared by Example 1 was first amplified by the polymerase chain reaction with NP1 and NP3 primers. 5 μl of the first reacted cDNA solution was continuously subject to the second polymerase chain reaction using NP2 and NP3 primers which contain recognition sites of *Bgl*II (AGATCT) and *Eco*RI (GAATTC) at 5' ends, respectively.

Example 3

Construction of expression plasmid pET-sNP

The process for the cloning of the nucleocapsid gene was depicted in Fig. 2. The two double-stranded DNA fragments amplified in Example 2 were characterized by agarose gel electrophoresis and then the DNA fragment containing 1.3 kb of nucleocapsid protein was recovered using GENECLEAN kit. The recovered DNA fragments were digested with restriction enzymes *Bgl*II and *Eco*RI, extracted once with a mixed solution of phenol and chloroform (1:1) and dissolved in 20 μl of sterile distilled water. Likewise, 5 μg of plasmid pET-3a as a cloning vector was digested with *Bgl*II and *Eco*RI, heated to 70℃ for 10 minutes in presence of 5 mM of EDTA, extracted once with a mixed solution of phenol and chloroform (1:1) and dissolved in 20 μl

of sterile distilled water. 5 μl of the vector was mixed with 10 μl of the restriction enzyme-cleaved nucleocapsid DNA and the ligation reaction was carried out at 25℃ for 3 hours in presence of T4 DNA ligase to form the desired expression plasmid pET-sNP.

Example 4

Construction of plasmid pKK-sNP

As primers, random 6-mers were used to synthesize cDNA from genomic RNA of Seoul virus R22. The first amplification of DNA was carried out by using the cDNA as template with NP1 and NP3 primers illustrated above. The second amplification of the DNA fragment was carried out by using the first amplified fragment as template with the following NP4 and NP5 primers which contain recognization sites of restriction enzymes *Eco*RI (GAATTC) and *Sal*I (GTCGAC) for cloning into pKK223-3, respectively:

NP 4 (23MER): 5'-CCGAATTCATGGCAACTATGGAG-3'
***EcoR* I**

NP 5 (23MER): 5'-GCGTCGACTTAGAGTTTCAAAGG-3'
***Sal* I**

The amplified nucleocapsid DNA fragment recovered from agarose gel was treated with Klenow enzyme to make both ends blunt and then the resulting blunt-ended DNA was treated with *Eco*RI to make one end cohesive. The vector pKK223-3 was digested with *Eco*RI and *Sma*I and ligated with the *Eco*RI-cleaved nucleocapsid gene. The resulting recombinant plasmid pKK-sNP

was introduced into E. coli JM105. It was found that pKK223-3 contains two *SalI* sites. This is why SmaI site was used instead of *SalI* for the construction of pKK-sNP.

Example 5

Production of transformed E. coli

An electric shock was used for the transformation and E. coli BL21(DE3) was used as a host cell for pET-sNP. 20 ml of LB medium was inoculated with a plate culture of the microorganism E. coli BL21(DE3) and a shake culture was carried out at 37℃ for 18 hours. 1 L of freshly made LB medium was inoculated with the first shake culture and a second shake culture was carried out at 37℃ until the optical density of 0.5-0.8 at 600 nm was reached. The resulting culture solution stood at 0℃ for 20 minutes and centrifuged to recover the cells. The recovered cell pellet was successively washed once with 1 L of cold sterile distilled water, once with 0.5 L of cold sterile distilled water and once with 20% glycerol. The cells were resuspended in 3 ml of 10% glycerol, divided into aliquots and kept at -70℃. Gene-Pulser (Bio-Rad) was used in electrical shock. A mixture of 40 μl aliquots of cells with 2 μl of DNAs was placed into cuvette with 0.2 cm of electrode gap. After one electric shock was pulsated on the cuvette following the adjustment of the Gene-Pulser at 25 μF of capacitance, 200 Ω of electric resistance and 12.5 kV/cm of electric field strength, immediately 1 ml of SOC medium (2% bactotrypton, 0.5% yeast extract, 10 ml NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) was added. After a shake culture at 37℃ for 1 hour, 0.1 ml and 0.9ml of cell cultures were sprayed on two LB medium agar plate with 50 μg/ml of ampicillin, respectively, and cultured in an incubator at 37℃ for 12 to 18 hours. A loop of cells picked from the agar plate medium was put into 80 μl of cracking buffer (0.05 M Tris, pH 6.8, 1% SDS, 2 mM EDTA, 0.4 M sucrose, 0.01% bromophenol

blue) and was made disperse by a vortex mixer. The disperse solution was centrifuged at 12,000 rpm for 15 minutes. The supernatant was subjected to an agarose gel electrophoresis to screen pET-sNP. The pET-sNP was reconfirmed by a digestion with restriction enzymes.

Example 6

Culture of transformant

The transformed cells were cultured overnight on LB medium (0.5% yeast extract, 1% bactotrypton, 1% NaCl, pH 7.0) with supplementary 0.5% glucose and 100 μg/ml of ampicilin. 1 L of the freshly made LB medium was inoculated with 5% overnight cell culture and was shaken at 37℃ with the velocity of 200 rpm. Once the optical density of 0.5 to 0.8 at A_{600} was reached, 0.1 to 2 mM IPTG was added to the culture solution and cultured for further 4 to 8 hours. The cells were recovered from the culture solution by centrifugation and washed once with 0.8% NaCl.

Example 7

Isolation and purification of nucleocapsid protein

A method for purifying the expressed nucleocapsid protein from a transformed E. coli with pET-sNP was illustrated in Fig. 3. The recovered cells were suspended in 50 to 100 ml of TE buffer solution (50 mM Tris, 1 mM EDTA, pH 8.0) and crushed by a sonicator. The sonication was continued until the concentration of the proteins in the crushed solution was no longer increased by a Bradford assay. The crushed solution was centrifuged at 8,000 x g for 1 hour. The amount of ammonium sulfate to be 25% to 50% of the saturated concentration was dissolved in the recovered supernatant. The resulting solution stood at room temperature for 1 hour and centrifuged at 8,000 x g for 30 minutes. The supernatant was discarded and the precipitate

was resuspended in 20 ml of TE buffer solution. The suspension was dialyzed twice in 2 L of TE buffer solution each for two hours to remove ammonium sulfate. The dialyzed solution was applied to gel filtration with Sepharcryl S200 (Pharmacia, Co), and then hydrophobic interaction chromatography with phenyl sepharose CL-4B resin (Pharmacia, Co). For loading of the samples, a solution of 40 mM KH_2PO_4/Na_2HPO_4 (pH 8.0) containing 0.6 M of ammonium sulfate was used, while, upon elution, a solution of 40 mM KH_2PO_4/Na_2HPO_4 (pH 8.0) without ammonium sulfate was used. Thereby, most nucleocapsid proteins were flown through, with most other proteins originated from *E. coli* being eliminated. The eluted nucleocapsid proteins were further dialyzed in PBS buffer solution and concentrated by Centricon or Centriprep (Amicon, Co.). All of the step-wise purified proteins were analyzed by polyacrylamide gel electrophoresis and western blotting (Fig. 4). The purity of final protein assumed to be higher than 90%.

Example 8

Western blotting of nucleocapsid protein

The purified proteins expressed from the transformed cells of the present invention were subjected to a polyacrylamide gel electrophoresis. The proteins on polyacrylamide gel were then transferred to nitrocellulose membranes and western blotting was carried out by using HFRS patient sera and anti-nucleocapsid protein monoclonal antibody ht9040. The membranes were reacted with 5% PBS solution (8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, 0.24 g KH_2PO_4, pH 7.4, per 1 L of PBS solution) containing skim milk for 30 minutes to permit only proteins having specificities to be adhered and then reatcted with the above monoclonal antibody ht9040 at room temperature for more than one hour. The membranes were washed three or four times with PBST solution (PBS + 0.5% Tween 20) each for 5 to 10 minutes to remove the unadhered antibodies. The washed membranes were

immersed into PBS solution containing 5% skim milk and appropriately diluted peroxidase-labelled anti-mouse immunoglobulin G and shaken for 1 hour. The membranes were washed further three or four times with PBST solution each for 5 to 10 minutes and stained with 4-chloro-1-naphtol. The desired nucleocapsid protein specific band (about 50 Kd) was exhibited on the expected spots (Fig. 5).

Example 9

Diagnostic assay on the purified nucleocapsid protein coated on 96-well plate by ELISA

An enzyme-linked immunoadsorbent assay (ELISA) was carried out to determine whether the Seoul virus nucleocapsid protein expressed by the transformed E. coli of the present invention is capable of recognizing HFRS patient sera. Twenty (20) HFRS patient sera and four (4) normal sera as control were tested. Each microtitre well in EIA/RIA 96-well plate was coated with 100 μl of solution of the nucleocapsid protein (100 ng/well) in coating buffer (50 mM $NaHCO_3$, pH 9.0) at normal temperature for 1-2 hours and washed twice with PBS buffer. 100 μl of patient or normal sera diluted with PBS was placed into each microtitre well and reacted for 1 hour. The plate was washed twice with PBST buffer and reacted with 1/1000 diluted peroxidase-labelled anti-mouse immunoglobulin G for 1 hour. Subsequently, the plate was washed twice with PBST buffer and 100 μl of 0.1 M citrate-phosphate buffer (pH 4.9) containing 1 mg/ml of OPD (o-phenylendiamine dihydrochloride) and 0.03% H_2O_2 was placed into each microtitre well. After the plate was kept at room temperature for 20 to 30 minutes in the dark, the reaction was stopped by placing 50 μl of 1 M sulfuric acid into each microtitre well and the absorbance at 490 nm was measured by ELISA Reader. The ELISA titres were defined as the reciprocal of the maximum dilutions of sera that generated absorbance readings higher

than 0.2. The results were shown in Table 3 below. It should noted from Table 3 that there are remarkable differences between ELISA titres of IgGs or IgMs from HFRS patients and those from normal humans with the expressed nucleocapsid protein. This indicates that ELISA using the expressed nucleocapsid protein of the present invention is very useful in the accurate diagnosis of HFRS. In order to enhance the accuracy of ELISA, all sera were subjected to indirect immunofluorescence assay. From Table 3, it should be noted that the results obtained by ELISA are completely consistent with those obtained by indirect immunofluorescence antibody assay.

Table 3

ELISA reactivities of HFRS patient sera and negative control human sera with the expressed nucleocapsid protein

Sample No.	Serum	ELISA(IgG)	ELISA(IgM)	IgG/IgM	*Indirect Immunofluorescence
1	Patient	8,000	1,000	8	+
2	Patient	8,000	<500	>16	+
3	Patient	8,000	2,000	4	+
4	Patient	64,000	1,000	64	+
5	Patient	8,000	500	16	+
6	Patient	1,000	4,000	0.25	+
7	Patient	1,000	<500	>2	+
8	Patient	1,000	<500	>2	+
9	Patient	4,000	2,000	2	+
10	Patient	8,000	1,000	8	+
11	Patient	1,000	<500	>2	+
12	Patient	2,000	500	>4	+
13	Patient	4,000	500	8	+
14	Patient	32,000	1,000	32	+
15	Patient	64,000	4,000	14	+
16	Patient	8,000	500	>16	+
17	Patient	500	1,000	0.5	+
18	Patient	8,000	4,000	2	+
19	Patient	1,000	<500	>2	+
20	Patient	16,000	8,000	2	+
21	Normal	100	50	2	-
22	Normal	100	50	2	-
23	Normal	400	50	8	-
24	Normal	100	50	2	-

* "+" and "-" signs represent test results positive and negative, respectively.

Example 10

Diagnostic assay by the use of the purified nucleocapsid protein-adhered nitrocellulose membrane

ELISA employing 96-well plate is useful in diagnosing many patients but requires the instrument ELISA reader. There is a need to conveniently diagnose a few HFRS patients occurred at rural or small areas.

The purified nucleocapsid proteins were diluted in PBS to make the

concentrations of 100, 50, 25, 13 and 6 μg/ml. The nitrocellulose membranes were immersed into the diluted solutions and the reactions were allowed by gently shaking it for 1 hour. The membranes were blocked by using PBS buffer solution with skim milk dissolved as 5%, dried at 30℃ for 4 hours and reacted with 1/500 dilution of patient sera in PBS buffer at normal temperature for 1 hour. Then, the membranes were washed twice with PBST solution (PBS, 0.5% Tween 20) and were reacted with 1/1000 dilution of second peroxidase-labelled anti-human antibodies in PBS for 1 hour. Subsequently, the membranes were washed twice with PBST solution and were stained with coloring agent [25 ml of 0.1 M Tris, pH 7.4, 25 mg of 4-chloro-1-naphtol (5 mg/ml in methanol), 3 μl of H_2O_2, 20 ml of distilled water]. When the color was appeared on the membranes, the reaction was allowed to be stopped and the membranes were washed with distilled water. It was observed that 13 μg/ml to 100 μg/ml of nucleocapsid protein solution resulted in the conspicuous appearance of the color by response with patient sera, while even 100 μg/ml of nucleocapsid protein solution did not result in the appearance of the color by respond with any normal human sera.

Table 4 below shows the serological reactivities of 100 μg/ml or 10 μg/ml of nucleocapsid protein solution adhered on nitrocellulose membranes with twenty patient sera and four normal human sera. All sera were affirmed by performing indirect immunofluorescence antibody assay.

Table 4

Sample Nos.	Serum	*Nucleocapsid protein		**Indirect immunofluorescence
		100 μg/ml	10 μg/ml	
1	Patient	++	+	+
2	Patient	++	++	+
3	Patient	++	++	+
4	Patient	+++	++	+
5	Patient	++	+	+
6	Patient	+	+	+
7	Patient	+	+	+
8	Patient	+	+	+
9	Patient	++	++	+
10	Patient	++	++	+
11	Patient	+	+	+
12	Patient	++	+	+
13	Patient	++	+	+
14	Patient	+++	++	+
15	Patient	+++	++	+
16	Patient	++	+	+
17	Patient	+	+	+
18	Patient	++	++	+
19	Patient	+	+	+
20	Patient	++	++	+
21	Normal	-	-	-
22	Normal	-	-	-
23	Normal	-	-	-
24	Normal	-	-	-

* Number of "+" signs represents the thickness of colors.

** "+" and "-" signs represent test results positive and negative, respectively.

Example 11

Assay on the reactivity of the purified nucleocapsid protein with HFRS vaccinee serum

Ten individuals were vaccinated with current commercially available

vaccine, Hantavax(Korean Green Cross, Co.) according to the vaccination schedule recommended by the producer, and were bled one month from the last vaccination. The reactivities of the purified nucleocapsid protein of Seoul virus R22 with pre-inoculation sera or post-inoculation sera were observed by performing similar procedures to Examples 8 and 9 except for using vaccinee sera instead of patient sera. The concentration of the nucleocapsid protein adhered on nitrocellulose membrane was 10 μg/ml. The results are shown in Table 5 below. It is noted from Table 5 that the results obtained by the use of nitrocellulose membrane are completely consistent with ELISA results. The results in Table 5 indicate that the purified nucleocapsid protein of the present invention is very useful in determining whether HFRS vaccine induced antibodies following its vaccinations.

Table 5

Vaccinees	ELISA		*Nitrocellulose Membrane	
	Pre-vaccination	Post-vaccination	Pre-vaccination	Post-vaccination
1	100	4,000	-	+
2	100	16,000	-	++
3	100	8,000	-	++
4	200	16,000	-	++
5	100	32,000	-	++
6	100	8,000	-	++
7	100	16,000	-	++
8	100	8,000	-	++
9	100	4,000	-	+
10	200	1,000>	-	-

* Number of "+" signs represents the thickness of colors, and "+" and "-" signs represent test results positive and negative, respectively.

Example 12

Assay on the efficacy of the vaccine prepared from the purified nucleocapsid protein

0.5 ml of the purified nucleocapsid protein in Example 7 was mixed with 0.625 mg of aluminum hydroxide gel as adjuvant and the mixture stood at 4℃ for 15 days. Thereafter, 0.01% (w/v) chimerosal and 0.02% gelatin were added to the mixture to prepare the final test vaccine.

Ginea pigs were used for the assay of the induction of neutralization antibodies of the vaccine. The current commercially available vaccine prepared by inactivating the virus cultured on rat brain was used as control. The concentrations of the antigen to be inoculated on the experimental animals were 10 μg/0.5 ml, 20 μg/0.5 ml and 40 μg/0.5 ml. The sera of guinea pigs inoculated with test vaccine and control vaccine were subjected to plaque reduction neutralization test to assay the immune efficacy of the vaccines. The plaque reduction neutralization test was performed as follows:

1) In order to obtain antibodies for the plaque reduction neutralization test, guinea pigs were subcutaneously inoculated three times at an interval of 10 days with three different concentrations of test vaccines and control vaccines (0.5 ml/inoculation).

2) Sera from guinea pigs were inactivated at 56℃ for 30 minutes and were diluted 1:10, 1:20, 1:40 and 1:80 in medium (MEM + M199 = 1:1) containing 3% fetal bovine serum.

3) The diluted sera were mixed with equal volumes of Hantaan virus 76-118 diluted to be 70 PFU/culture vessel (diameter of 6 cm) in test tubes and reacted at 37℃ for 1 hour.

4) The preparative monolayer of Vero E6 cell in 6 cm culture vessel was inoculated with 0.2 ml of the above mixed solution and the reaction was allowed at 37℃ for 90 minutes.

5) Inoculated medium was removed and 5 ml of the first agarose overlay was formed on each culture vessel. The composition of the first agarose overlay is as follows:

M199 medium	50 ml
Fetal bovine serum	10 ml
7% $NaHCO_3$	3 ml
Agarose	0.8 g
Distilled water	50 ml

The M199 medium contains no phenol red and the fetal bovine serum was heated to 56℃ for 30 minutes.

6) After the first agarose overlay was completed, inoculated cells were cultured at 37℃ for 10 to 11 days.

7) 3 to 3.5 ml of the second agarose overlay was formed on each culture vessel. The composition of the second agarose overlay is as follows:

M199	50 ml
400 mM MES*	10 ml
5% BSA	5 ml
0.6% Neutral Red	1 ml
1N NaOH	1.5 ml
Agarose	0.7 g
Distilled Water	32.5 ml

*(2-(N-Morpholino)ethane sulfonic acid, monohydrate)

8) After the second agarose overlay was completed, it was cultured for 3 days and the number of plaques with diameter of about 2 mm was counted.

Table 6

Serum	Serum Dilutions	Number of Plaques(PFU)
Normal	1:10	72
	1:20	74
	1:40	69
	1:80	74
Test Vaccine	1:10	8(40 μg), 12(20 μg), 19(10 μg)
	1:20	14(40 μg), 22(20 μg), 31(10 μg)
	1:40	16(40 μg), 32(20 μg), 42(10 μg)
	1:80	25(40 μg), 38(20 μg), 64(10 μg)
Control Vaccine	1:10	23
	1:20	31
	1:40	41
	1:80	63

It should noted from Table 6 above that the induction of neutralization antibodies of the test vaccine prepared from the purified nucleocapsid protein at concentration of 40 μg/0.5 ml and 20 μg/0.5 ml are much higher than the control vaccine, while the inductions of neutralization antibodies of the test and control vaccines at concentration of 10 μg/0.5 ml are equal. The results indicate that the vaccine prepared from the nucleocapsid protein of Seoul virus R22 according to the present invention exhibits excellent immunogenicity against HFRS as compared with the current available vaccines. Especially, since the vaccine of the present invention is prepared from the single nucleocapsid protein, rather than the virus *per se* which is believed to include toxical materials, it is apparent that the vaccine of the present invention is very superior to the current available vaccines in terms of safety.

WHAT IS CLAIMED IS:

1. A Seoul virus R22 nucleocapsid gene having the following nucleotide sequence:

```
          10         20         30         40         50
          |          |          |          |          |
ATGGCAACTATGGAGGAAATCCAGAGAGAAATCAGTGCTCACGAGGGGCA

          60         70         80         90         100
          |          |          |          |          |
GCTTGTGATAGCACGCCAGAAAGTCAAGGATGCAGAAAAGCAGTATGAAA

          110        120        130        140        150
          |          |          |          |          |
AGGATCCTGATGACTTCAACAAGAGGGCACTGCATGATCGGGAGAGTGTC

          160        170        180        190        200
          |          |          |          |          |
GCAGCTTCAATACAATCAAAAATTGATGAATTGAAGCGCCAACTTGCCGA

          210        220        230        240        250
          |          |          |          |          |
CAGGATTGCAGCAGGGAAGAATATTGGGCAAGACCGGGATCCTACAGGGG

          260        270        280        290        300
          |          |          |          |          |
TAGAGCCGGGTGATCATCTCAAAGAGAGATCAGCACTAAGCTATGGGAAT

          310        320        330        340        350
          |          |          |          |          |
ACACTGGACCTGAATAGTCTTGACATTGATGAACCTACAGGACAGACAGC

          360        370        380        390        400
          |          |          |          |          |
TGATTGGTTGACCATAATTGTCTATCTGACTTCATTTGTGGTCCTGATCA

          410        420        430        440        450
          |          |          |          |          |
TCTTAAAGGCACTGTACATGTTAACAACAAGAGGCAGGCAGACTTCAAAG

          460        470        480        490        500
          |          |          |          |          |
GACAACAAAGGGATGAGGATCAGATTCAAGGATGACAGCTCATATGAGGA
```

```
          510         520         530         540         550
TGTCAATGGAATCAGAAAACCCAAACATCTGTATGTGTCAATGCCAAACG

          560         570         580         590         600
CCCAATCCAGCATGAAGGCTGAAGAGATAACACCTGGAAGATTCCGCACT

          610         620         630         640         650
GCAGTATGTGGGCTATATCCTGCACAGATAAAGGCAAGGAACATGGTGAG

          660         670         680         690         700
CCCTGTCATGAGTGTAGTTGGGTTTTTGGCACTGGCAAAAGATTGGACAT

          710         720         730         740         750
CTAGAATTGAAGAATGGCTTGGTGCACCCTGCAAGTTTATGGCGGAATCT

          760         770         780         790         800
CCAATTGCTGGGAGTTTATCTGGGAATCCCGTGAATCGTAACTATATCAG

          810         820         830         840         850
ACAGAGACAAGGTGCACTTGCAGGGATGGAGCCAAAGGAATTTCAAGCCC

          860         870         880         890         900
TCAGGCAACATGCAAAGGATGCTGGGTGTACACTGGTTGAGCATATTGAG

          910         920         930         940         950
TCACCATCATCAATATGGGTGTTTGCTGGGGCCCCTGATAGGTGTCCACC

          960         970         980         990        1000
AACATGTTTGTTTGTCGGAGGGATGGCTGAGTTAGGTGCCTTCTTTTCTA

         1010        1020        1030        1040        1050
TACTTCAGGATATGAGGAACACAATCATGGCTTCAAAAACTGTGGGCACA
```

```
          1060       1070       1080       1090       1100
GCTGATGAAAAGCTTCGAAAGAAATCATCATTCTATCAATCATACCTCAG

          1110       1120       1130       1140       1150
ACGCACACAATCAATGGGAATACAACTGGACCAGAGGATAATTGTTATGT

          1160       1170       1180       1190       1200
TTATGGTTGCCTGGGGAAAGGAGGCAGTGGACAACTTTCATCTCGGTGAT

          1210       1220       1230       1240       1250
GACATGGACCCAGAGCTTCGTAGCCTGGCTCAGATCCTGATTGACCAGAA

          1260       1270       1280       1290
AGTAAAAGAAATCTCGAACCAGGAACCTTTGAAACTCTAA
```

2. A recombinant expression plasmid containing the Seoul virus R22 nucleocapsid gene according to claim 1.

3. The recombinant expression plasmid according to claim 2, which is the expression plasmid pET-sNP constructed by cloning the Seoul virus R22 nucleocapsid gene of claim 1 into vector pET-3a.

4. A transformed *Escherichia coli* with the recombinant expression plasmid according to claim 2.

5. The transformed microorganism according to claim 4, which is the transformed E. coli BL(pET-sNP) produced by introducing the expression plasmid of claim 3 into E. coli BL21(DE3).

6. A fused form of Seoul virus R22 nucleocapsid protein having the following amino acid sequence isolated and purified from culture of the transformed

microorganism of claim 4:

```
          10         20         30         40         50
          |          |          |          |          |
MASMTGGQQMGRGSMATMEEIQREISAHEGQLVIARQKVKDAEKQYEKDP

          60         70         80         90         100
          |          |          |          |          |
DDFNKRALHDRESVAASIQSKIDELKRQLADRIAAGKNIGQDRDPTGVEP

          110        120        130        140        150
          |          |          |          |          |
GDHLKERSALSYGNTLDLNSLDIDEPTGQTADWLTIIVYLTSFVVLIILK

          160        170        180        190        200
          |          |          |          |          |
ALYMLTTRGRQTSKDNKGMRIRFKDDSSYFEDVNGIRKPKHLYVSMPNAQS

          210        220        230        240        250
          |          |          |          |          |
SMKAEEITPGRFRTAVCGLYPAQIKARNMVSPVMSVVGFLALAKDWTSRI

          260        270        280        290        300
          |          |          |          |          |
EEWLGAPCKFMAESPIAGSLSGNPVNRNYIRQRQGALAGMEPKEFQALRQ

          310        320        330        340        350
          |          |          |          |          |
HAKDAGCTLVEHIESPSSIWVFAGAPDRCPPTCLFVGGMAELGAFFSILQ

          360        370        380        390        400
          |          |          |          |          |
DMRNTIMASKTVGTADEKLRKKSSFYQSYLRRTQSMGIQLDQRIIVMFMV

          410        420        430        440
          |          |          |          |
AWGKEAVDNFHLGDDMDPELRSLAQILIDQKVKEISNQEPLKL
```

7. A pharmaceutical composition for diagnosis of HFRS comprising the fused form of Seoul virus R22 nucleocapsid protein of claim 6.

8. A vaccine for prevention of HFRS comprising the fused form of Seoul virus R22 nucleocapsid protein of claim 6.

FIG. 1

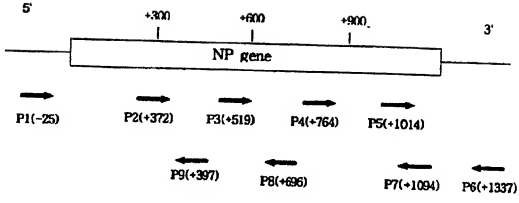
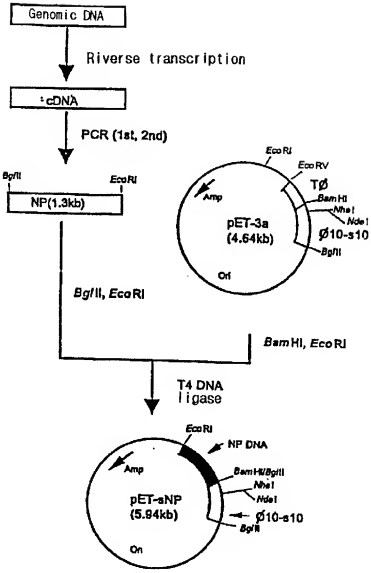

5'
+300
+600
+900
3'
NP gene
P1(-25)
P2(+372)
P3(+519)
P4(+764)
P5(+1014)
P9(+397)
P8(+696)
P7(+1094)
P6(+1337)

FIG. 2


Genomic DNA
Riverse transcription
cDNA
PCR (1st, 2nd)
BglII
EcoRI
NP(1.3kb)
EcoRI
EcoRV
TØ
BamHI
NheI
NdeI
Ø10-s10
BglII
Amp
pET-3a
(4.64kb)
Ori
BglII, EcoRI
BamHI, EcoRI
T4 DNA
ligase
EcoRI
NP DNA
Amp
BamHI/BglII
NheI
NdeI
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BglII
pET-sNP
(5.94kb)
On

FIG. 3

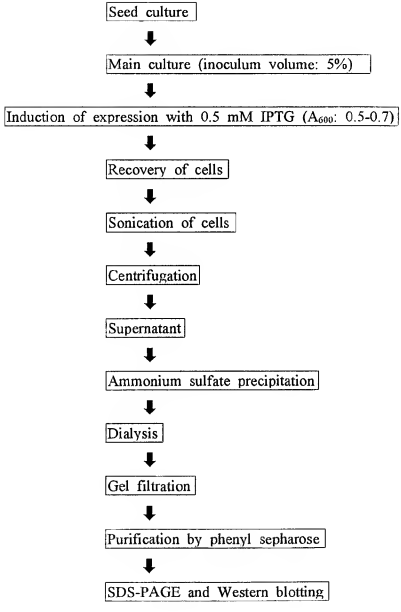

Seed culture
Main culture (inoculum volume: 5%)
Induction of expression with 0.5 mM IPTG (A_{600}: 0.5-0.7)
Recovery of cells
Sonication of cells
Centrifugation
Supernatant
Ammonium sulfate precipitation
Dialysis
Gel filtration
Purification by phenyl sepharose
SDS-PAGE and Western blotting

FIG. 4

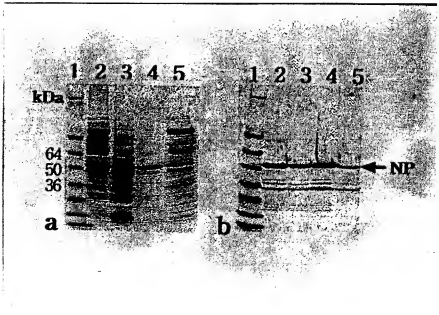


FIG. 5

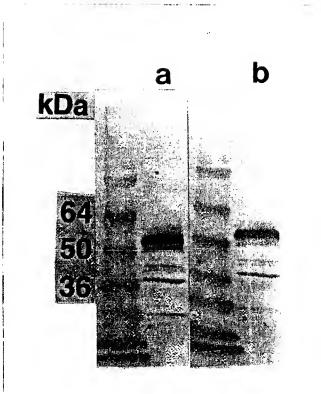


FIG. 6

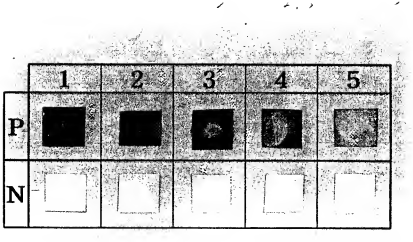
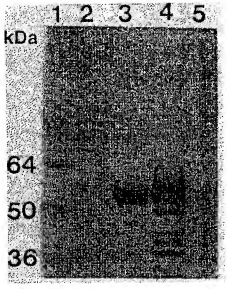


FIG. 7



INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR 97/00184

A. CLASSIFICATION OF SUBJECT MATTER

IPC[6]: C 12 N 15/33; A 61 K 38/16

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC[6]: C 12 N 15/33; A 61 K 38/16

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	THE JOURNAL OF GENERAL VIROLOGY, Vol.74, April 1993, W. WANG et al.:"Expression of non-conserved regions of the S genome segments of three hantaviruses: evaluation of the expressed polypeptides for diagnosis of haemorrhagic fever with renal syndrome", pages 1115-1124, totality.	1-4
A	Database WPIL on Questel, week 01, London, Derwent Publications Ltd., AN 96-008501, KR 9402012 B1, abstract.	1

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 December 1997 (12.12.97)	05 January 1998 (05.01.98)
Name and mailing address of the ISA/ AT AUSTRIAN PATENT OFFICE Kohlmarkt 8-10 A-1014 Vienna Facsimile No. 1/53424/535	Authorized officer Wolf Telephone No. 1/53424/436

Form PCT/ISA/210 (second sheet) (July 1992)

PCT/KR97/00184

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

To. CHEILJEDANG Corporation
12F CHEIL BLDG, 500,5GA,
NAMDAEMOON-NO, CHUNG-KU,
SEOUL, 100-095 KOREA

RECEIPT IN THE CASE OF AN ORIGINAL issued pursuant to Rule 7.1 by the INTERNATIONAL DEPOSITARY AUTHORITY identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR : E. coli BL(pETSNP)	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY : KCCM - 10111
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I above was accompanied by: ☐ a scientific description ☐ a proposed taxonomic designation (Mark with a cross where applicable)	
III. RECEIPT AND ACCEPTANCE	
the microorganism identified under I above was received by this international Depositary Authority on Sep. 16, 1996 and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on Sep. 10, 1997.	
IV. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: Korean Culture Center of Microorganisms Address: Department of Food Engineering College of Eng. Yonsei University Sodaemun-gu, Seoul 120-749 Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s) : Date: Sep. 19. 1997

Form BP/4 (KCCM Form 17)

Sole page